Molecular alterations induced by viral pathogen in crustacean cells

FINAL SYNOPSIS

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Final synopsis

Introduction

Viruses are abundant in water and hence viral diseases are quite common among aquatic organisms. The diseases create great problem to the commercial shrimp farming. At present the most common and most disastrous disease of shrimp is caused by white spot syndrome virus (WSSV), which has spread all over the world and is infecting many different species of crustaceans (Jiravanichpaisal 2005). WSSV is pathogenic to at least 78 species, mainly the decapod crustaceans including marine and freshwater shrimp, crab, crayfish and lobsters (Lightner 1996; Flegel 2006).

Viruses are very simple entities containing only nucleic acid, a protein coat and in some cases a membrane or envelope derived from the host cell membranes (Johnson *et al*., 2008). WSSV is an enveloped DNA virus that causes 100% mortality in cultured shrimp within 3–10 days (Lightner 1996). This virus is the only member of the family *Nimaviridae*, genus *Whispovirus* (Mayo 2002). Due to the extreme virulence of WSSV, and wide range of host covering almost all crustaceans (Lo *et al*., 1996a; Chen *et al*., 1997; Sahul Hameed *et al*., 2001; Sahul Hameed *et al*., 2003), it is difficult to prevent the spread of the virus. Although considerable progress has been

made in the characterization of WSSV, the level of our understanding of the shrimp defense system in response to the viral infection is still poor. Knowledge of the response of shrimp immune system consequent to viral infection is necessary to develop control methods and minimize the loss of production due to infectious diseases (Wongpanya *et al*., 2007).

The invertebrate immune response to viruses is particularly poorly understood. It is well known that shrimp, like other crustaceans and invertebrates lack a truly adaptive immune system and appear to rely on a variety of innate immune response systems to recognize and destroy ‘‘non-self’’ materials (Lee and Soderhall 2002). Hemocytes are believed to be the primary site of shrimp immune responses where several immune reactions take place (Bachere 2003). It has been shown that the shrimp which recover from viral infection contain a humoral neutralizing factor that can reduce the virus (Wu and Muroga 2004). Several proteins and peptides are synthesized and stored in haemocytes and released into the haemolymph upon infections (Sritunyalucksana and Soderhall 2000).

Recently, several innate immune response systems have been characterized in crustaceans, and a number of genes that are involved in the shrimp immune system have been identified and characterized (Gross *et al*., 2001; Dhar *et al*., 2003; Luo *et al*., 2003; Pan *et al*., 2005; Rojtinnakorn *et al*., 2002; Tassanakajon *et al*., 2006). Using expressed sequence tag analysis, the immune-related genes in haemocytes have been identified from two sister species of Litopenaeid shrimps: Pacific white shrimp (*Litopenaeus vannamei*) and Atlantic white shrimp (*L. setiferus*) (Gross *et al*., 2001) and other shrimps viz. *Marsupenaeus japonicus* (Rojtinnakorn *et al*., 2002), *Penaeus monodon* (Supungul *et al*., 2002) and *Fenneropenaeus chinensis* (Shen *et al*., 2004).

The initial viral load in the infected shrimp tissues is known to be one of the most important factors in the rate of progression and transmission of the disease. Because of the lack of continuous *in vitro* cell cultures for shrimp, the quantification of WSSV particles has not been possible (Durand and Lightner 2002). PCR-based methods especially the quantitative methods can be used for viral quantification in shrimp tissues. The techniques such as competitive PCR and real-time PCR permit the determination of the number of copies of a target segment of DNA in a particular tissue sample. These methods have been applied to penaeid shrimp virology and to develop assays for WSSV and IHHNV (Tang and Lightner, 2000, 2001). Quantitative detection

methods can reveal the severity of a WSSV infection in a culture pond, and thus could be a useful tool to determine whether to harvest (if the infection is severe) or to continue with remedial approaches if the infection is light (Lo *et al*. 1998).

Viral infection cycles involve numerous protein-protein interactions between virus and host proteins. These interactions range from the initial binding between host membrane receptor and viral coat proteins to take-over the host transcription machinery by replacing specificity-determining host factors by viral proteins. Most of these interactions are essential for viral propagation and can be potential targets for anti viral therapy (Huber *et al*., 1994). A better understanding of host response to WSSV will help to elucidate the pathogen's unique mechanisms of virulence and pathogenesis (Wang *et al*., 2007). To understand the pathogenesis of any disease, knowledge of the interactions between virus and host is critical. Virus-host interactions may result in immune responses against the invader, and may also result in changes in the expression levels of host genes that favor virus replication (Wang *et al*., 2007). To date, virus–host interactions of WSSV have been studied only at the transcription level using expressed sequence tags (ESTs), RT-PCR, microarray chips, suppression subtractive hybridization and differential hybridization (Astrofsky *et al*., 2002; Bangrak *et al*., 2002; Dhar *et al*., 2003; Gross *et al*., 2001; He *et al*., 2005; Pan *et al*., 2005; Rojtinnakorn *et al*., 2002; Roux *et al*., 2002). However, though many of these studies which were focused on immune cells (lymphoid organ cells and hemocytes) has provided good insights into biodefense mechanisms, these cells are not the primary WSSV targets (Lo *et al*., 1997; Tsai *et al*., 1999; Wu and Muroga 2004), and therefore, their cellular pathways are not necessarily representative of the host cells in which virus replication occurs (Wang *et al*., 2007). In consequence, little is known about the cellular events associated with WSSV infection in permissive cells. Identifying the changes in the protein profile consequent to WSSV infection is an important first step toward improving our understanding of the cellular pathways involved in the WSSV infection (Wang *et al*., 2007).

Little is known about WSSV infection and morphogenesis *in vivo*. Research on virus replication and virion morphogenesis shows that DNA replication and *de novo* envelope formation take place in the nucleus (Durand *et al*., 1997; Wang *et al*., 2000). Understanding the interaction between host and pathogen will be helpful in developing control strategies for infectious diseases in shrimp. Although an increasing number of immune function related genes of shrimp have

been reported, the genes involved in the interaction between WSSV and shrimp still remain unclear (Wang *et al*., 2006).

The gills are one of the primary target tissues of WSSV infection (Lo *et al*., 2004). Gills serve for respiration, osmoregulation, excretion and acid base homeostasis (Taylor and Taylor 1992). The Histopathological studies on WSSV infected *P. monodon* have shown massive tissue disintegration in the gills, stomach, lymphoid organ, heart, midgut, hindgut and nervous tissue (Rajan *et al*., 2000). Moreover, large amount of WSSV in gill, stomach, midgut and muscle were observed upon infection (Lo *et al*., 1996b).

The hepatopancreas, a digestive organ in crustaceans that performs functions similar to those of the liver and pancreas in vertebrates, is crucial in the immune system of penaeid shrimp (Pan *et al*., 2005)**.** The shrimp hepatopancreas has been used as a monitor organ for the overall assessment of the health and well-being of the intact organism. Hepatopancreas functions are important to the health, growth and survival of cultured shrimp (Hu and Leung 2007). Understanding the protein profile changes in the tissues, especially those tissues which are the primary targets of WSSV is an important step in understanding the host pathogen interaction during WSSV infection. However, information about the proteins that may be involved in WSSV pathogenesis remains limited. Recently, though considerable progress has been made in the detection of genes related to WSSV infection (Zhao *et al*., 2007; Leu *et al*., 2007; Pan *et al*., 2005), studies on the time dependent functional expression of these genes are very meager.

Objective of the Study

White spot syndrome virus cause huge production losses to most of the shrimp producing countries of the world. Researchers have tested several control strategies, but with limited success. Proper understanding of the defense system in the wake of viral infection is essential for developing effective control strategies.

There are always a small number of animals surviving after an outbreak of WSS disease. These survivors possess stronger defense ability that enables them to survive the disaster. There must be qualitative and quantitative variations in gene expression of these shrimps compared to those

succumbing to the infection. Of late, attention is being paid to the immune related genes of shrimp. Within an individual itself, the gene expression pattern can vary in a time dependent manner, consequent to the viral progression. Studies by Leonardo *et al* (2005) indicated that a number of genes are involved in the defense response of *P. monodon*. However, these studies were mainly on the gene expression late during the infection (1-3 days). There is only limited information available about the host gene response during the initial hours of infection.

Interactions between virus and host at cellular protein level is part of the viral propagation and the immune responses of the host against the invader. Some of these proteins result from the modulations of the expression of host genes that are activated during virus replication. Understanding the host response to WSSV will help to elucidate the mechanisms of virulence and pathogenesis, and shall be of help in developing anti viral therapy.

Therefore, the present study is directed mainly towards evaluation of the expression of seven immune related genes in *P. monodon* in the early phase of WSSV infection, in addition to the protein expression variation and the rate of viral progression in different tissues, in a time dependant manner, following the infection.

The main goals of the study were

1) Assessing the WSSV copy number in different tissues during viral progression following experimental infection.
2) Studying the immune gene expressional variations in the host during viral progression.
3) Correlating the immune gene expression with the survivability of the infected animals.
4) Understanding the protein expressional variation during viral progression.

Summary of Chapters

The research findings are presented in three separate chapter's viz. Quantification of viral replication in the host animal, Differential expression of immune related genes and Protein expressional variations following experimental infection of *P. monodon* with WSSV.

The **chapter one** deals with viral progression rate in shrimp after experimental infection with known copy number of WSSV. Shrimps (*Penaeus monodon*) of sub adult stage which were tested to be negative to WSSV through PCR were used for the experiment. Animals in the intermoult stage or early premoult stage were used for the study. For the viral infection, all the animals except the control were injected with the viral inoculum (50 µl, with a known copy number) between the second and third tergal plates of the lateral side of the tail using a 1ml syringe (30G).

After injection with the WSSV, the infected shrimps showed obvious signs of infection. At 24 hrs post-injection (hpi), the shrimps displayed very little feeding and a faded or reddish body color. By 65 hpi, some of the infected shrimps were dead whereas the remaining ones appeared weak to moribund. Before 105 hpi all the infected shrimps were dead.

Five infected shrimps were selected at each time point (12, 24, 36 and 48 hours) after inoculation and tissues like gill, muscle, pleopod and hemocyte were collected from each shrimp and flash frozen at -80 ^{0}C in a deep freezer. Hemolymph was collected using a 30G needle fitted to a 1-ml syringe pre filled with 100 µl anticoagulant (Alsevers solution) and centrifuged at 3000 rpm for 10 minutes to collect the hemocyte pellet.

Respective tissues from all the 5 animals belonging to each time point were pooled and DNA was extracted separately from gills, muscle, pleopod and hemocytes using Qiagen DNeasy Kit. The DNA thus extracted is expected to contain both the host DNA and viral DNA.

The viral progression following viral inoculation was estimated from the viral copy number in the tissues at different time points following the inoculation. Viral copy number was estimated using SYBR green real time PCR. The primers used for the PCR amplification of WSSV DNA were developed from a region of WSSV genomic sequence available in the GenBank database.

The primers were designed using the Beacon designer version 7.51 (Premier Biosoft International, CA, USA).

To generate plasmid standards for real-time PCR, the PCR amplified target sequence was cloned into a vector (TOPO TA Cloning Kit, Invitrogen, Germany). The plasmid DNA concentration was determined using spectrophotometer.

The copy number of this recombinant plasmid was calculated with the following formula:

Plasmid size [bp] = vector size [bp] + insert size [bp]
1 bp dsDNA correspond to 660 g/mol
1 mol = 6.023×10^{23} mol^{-1}(Avogadro constant)
Plasmid size [bp] × 660 g/mol = molarity [g/mol]
Molarity [g/mol] / 6.023×10^{23} = weight [g/plasmid or ng/plasmid]
DNA concentration [ng/µl] / weight [ng/plasmid] = n plasmid [plasmid/µl]

Real time PCR assay of the DNA extracted from the infected shrimp tissues, at different time points, using WSSV specific primers were carried out to quantify the viral copy number in them. The SYBR Green based real-time PCR assay was carried out in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, California). The amplifications were performed in 96-well plates in 25 µl reaction volume containing 12.5 µl of 2x iQ SYBR Green Supermix (Bio-Rad), 0.5 µl (each) of the forward and reverse primers (5 µM), 1 µl of template and 10.5 µl of water. The thermal profile for the SYBR Green real-time PCR was 95 ^{0}C for 4 min followed by 40 cycles of 95 ^{0}C for 30 s and 51 ^{0}C for 30 s and extension at 72 ^{0}C for 30 s. After amplification, a melting-curve analysis was conducted to ensure that a single product had been amplified. In a 96-well plate, each sample was run in triplicate. DEPC-water replacing the template was used as the negative control. A series of dilutions of WSSV recombinant plasmid from 1.32×10^1 – 1.32×10^6 were used for generating standard curve. After the PCR program, data were analyzed with SDS 2.4 software (Applied Biosystems).

WSSV virus DNA was detected in all the *P. monodon* tissues under study using real-time PCR. The sample copy numbers were determined against a standard curve generated by serial dilution of the plasmid. The viral genomic DNA which is equivalent to virus genome equivalents of

replicating virus were determined. The viral load in the tissues increased as the time progressed. The viral copy number of tissues at different time intervals were analyzed by one-way analysis of variance (ANOVA), showed a significant difference between tissues at all time periods evaluated ($P < 0.001$). Subsequent pairwise analysis of WSSV copy number in different tissues at a particular time interval by Tukey post-hoc test revealed a high level of significance ($P < 0.001$).

In the gill, 8.8×10^2 WSSV DNA copies/µg of total DNA were detected at 12 hpi and levels continued to increase to 6.28×10^5, 3.25×10^6 and 6.45×10^8 at 24, 36 and 48 hpi. Compared to gills, WSSV DNA levels in pleopod showed a 10 fold increase at the first sampling (2.6×10^3 WSSV DNA copies/µg of total DNA at 12hpi), though at the later stages it came down (1.35×10^5, 2.7×10^6 and 3.2×10^8 WSSV DNA copies/µg of total DNA at 24, 36 and 48 hpi respectively). Though the WSSV DNA copies (copies/µg of total DNA) in the muscle were similar to pleopod at 12 hpi (2.56×10^3) it was about 10 fold lower at 24 and 36 hpi (3.9×10^4 and 8.55×10^5), and about 100 fold lower at 48 hpi (7.8×10^6). In the hemocytes the WSSV DNA level was 4.3×10^3 at 12 hpi and a marginal increase in progression was observed along with time (9.5×10^3, 2×10^4 and 1.8×10^6 WSSV DNA copies/µg of total DNA at 12, 24, 36 and 48 hpi, respectively).

The rate of viral progression is found to vary in the tissues under study viz. gill, hepatopancreas, muscle and hemocyte. It has been reported that gill is the main target of WSSV, and this study also confirms it with the highest viral load in gills compared to other tissues. At 48 hpi the virus copies in the muscle and hemocytes were almost 100 times less than the gills and pleopod.

The **chapter two** describes the differential expression of the immune related genes during WSSV infection in hemocytes. For the gene expression studies shrimps were inoculated with WSSV, and hemolymph (100 µl) was collected from four animals each at 3, 6, 12, 24, 36 and 48 hrs post inoculation using 30G needle fitted to a 1-ml syringe pre filled with 100 µl anticoagulant (Alsevers solution). Hemolymph was centrifuged at 3000 rpm for 10 minutes to collect the hemocyte pellet. Total RNA from the hemocytes was isolated using RNA isolation kit (MACHERY-NAGEL GmbH & Co, Germany) wherein genomic DNA contamination is removed by on-column DNase treatment according to manufacturer's protocol. RNA was

quantified by spectrophotometer and its quality was assessed by electrophoresis on 1% agarose gel. The cDNA was synthesized using total RNA from each sample, through reverse transcription using a Bio-Rad iscript cDNA kit. Presence of the transcripts of the genes under study viz. Prophenoloxidase-1, Prophenoloxidase-2, Hemocyanin, Crustin, Penaeidin and C-lectin and Lysozyme was investigated through PCR amplification of the cDNA using gene specific primers. Primers for Prophenoloxidase-1, Prophenoloxidase-2, Hemocyanin and Lysozyme were designed from the published sequences in NCBI (AF099741, FJ025814, AF41737, AF539466) using the Beacon designer version 7.51 (Biosoft International, CA, USA). The published primers were used for Crustin, Penaeidin and C-lectin.

To quantify the changes in expression level of the genes during the progression of WSSV namely at 3, 6, 12, 24, 36 and 48 hrs post inoculation, relative transcription levels of the genes under study were estimated Semi-quantitatively. For this, PCR amplification of cDNA was carried out using the gene specific primers. Elongation factor 2 (Lehnert *et al.*, 1999), a housekeeping gene, was used as an internal control for all experiments.

PCR was performed with 1µl of $1/5^{th}$ diluted cDNA in 25µl reaction volume, containing 1x Standard Taq buffer, 0.2 µM each dNTPs, 0.2 µM each primer and 1 U Taq DNA polymerase (Sigma Aldrich). The cycling parameters used were as follows: an initial denaturation at 95 ◦C for 4 mins, followed by 28-35 cycles (depending on the gene) at 95 ◦C for 30s, 60 ◦C for 30s, 72 ◦C for 30s and a final extension at 72 ◦C for 5 min. Ten microlitres of PCR products were analyzed by electrophoresis using 2% agarose gel in TBE buffer, stained with ethidium bromide and visualized under a UV-transilluminator. For quantification of the amplified product, the intensity of the gel bands were measured using Quantity One (Bio-rad) analysis software. The duration of survival of each animal after the inoculation was recorded to correlate it with the gene expression level.

Expression of all the genes under study viz. Hemocyanin, Prophenoloxidase-1, Prophenoloxidase-2, Crustin, C-lectin, Lysozyme and Penaeidin were found to be affected by the WSSV infection. Differential expression was noticed, while most of them were up regulated, two were found to be down regulated. The significance of expression of all genes under study were statistically evaluated. The Penaeidin in this study showed an upregulation from 3^{rd} hr onwards and reached maximum by 24 hpi. Then it declined and at 48 hpi, the expression level was lower

than the control animals. The Lysozyme and C-lectin showed an upregulation of expression from 3rd hr onwards and reached the maximum level at 12 hpi ($P < 0.05$). Thereafter, though they were slowly downregulated, the level of expression was much higher than the control animal. However Crustin showed downregulation right from the initial hours and the level of expression remained lower than the control animals.

Within the PO system the expressional variation of Prophenoloxidase and Hemocyanin were evident. While both the Prophenoloxidase homologues (proPO-1 and proPO-2) started showing early upregulation, the hemocyanin showed an initial downregulation. Expression of proPO-1 showed increase from 3 hpi, reaching maximum at 12 hpi ($P < 0.05$) and continued to be so up to 24 hpi. The proPO-2 showed an upregulation up to 24 hpi and then was downregulated. Hemocyanin in this study showed an downregulation from the initial hours. Though it showed an upregulation at 24 hrs it was short lived.

On the premise that the animals with higher expression may have better defense ability which enable them to survive for long on infection, an attempt was made to correlate the gene expression level with the survival period, of the animals experimentally infected with WSSV. The gene expression level at 24 hpi was correlated with the survival. Those animals which survived beyond 84 hrs (3½ days) were found to have a higher level of expression of Lysozyme, C-lectin, Prophenoloxidase-1 and Prophenoloxidase-2 than those which died before 66 hrs (2 ½ days) except in the case of Penaeidin.

The **chapter three** describes the protein expressional variations during WSSV progression in different tissues. Changes in the profile of total protein, nuclear protein and mitochondrial proteins, following WSSV infection was monitored in this study. For this, tissues like hepatopancreas, muscle and gills were then excised from infected and control shrimps at 6, 12, 24, 36 and 48 hour post-injection (hpi) and flash frozen at -80 ^{0}C for protein expression studies. To minimize deviations arising from individuals, the sample from five individuals were pooled for each time point, for both the control and WSSV injected samples. The total proteins, mitochondrial proteins and nuclear proteins were isolated from these tissues and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out.

For total protein isolation, hundred milligrams of each tissue was homogenized in TN buffer (20 mM Tris –HCl, 400 mM NaCl, pH 7.4) in cold conditions and centrifuged at 4 ^{0} C for 10,000 rpm for 20 minutes. The supernatant containing total protein was preserved at -80 ^{0}C.

For isolation of nuclear and mitochondrial proteins two hundred milligrams of tissues were minced and homogenized in 0.25 M sucrose and 0.0018 M calcium chloride under ice cold condition. The homogenate was loaded in a centrifuge tube containing sucrose gradient (0.034 M sucrose and 0.00018 M Calcium chloride) solution and centrifuged at 2000 rpm for 10 minutes at 4 ^{0}C. The fraction settled above the gradient was saved for mitochondrial protein isolation and pellet for nuclear protein isolation.

The nuclear pellet was dissolved in 10% SDS prepared in 0.025 M sucrose and 0.00018 M calcium chloride and kept at room temperature for 15 minutes and then stored at -80 ^{0} C. The mitochondrial fraction was centrifuged at 10,000 rpm for 20 minutes at 4 ^{0} C. The resulting pellet was dissolved in 10% SDS prepared in 0.025 M sucrose and 0.00018 M calcium chloride and kept at room temperature for 15 minutes and then stored at -80 ^{0}C.

The protein profile was developed by the procedure of discontinuous, denaturing gel electrophoresis (SDS-PAGE) performed in a Tris-glycine buffer system with 10% separating gel and 5% stacking gel according to Laemmli *et al*., 1970. Proteins were separated according to their molecular weights.

The SDS PAGE apparatus was assembled according to the manufacturer's instruction manual. Before loading, protein samples were mixed with loading buffer in equal ratio and boiled for 5 minutes to reduce the disulfide bonds. The gel was run at voltage of 65 V in separating gel, and then gradually increased to 140 V in stacking gel, until the bromophenol blue reached the bottom of the gel. Thereafter, the gel was fixed. Then the gel was stained with Coomassie staining solution for one hour, and then destained with the destaining solution containing 10% methanol and 7% acetic acid. A known protein molecular weight marker mix (Bio-Rad), with proteins ranging from 14.4 to 97.4 kDa, was co-electrophoresed to determine the molecular weights of the proteins. The molecular weights of the proteins resolved through the electrophoresis were identified using Quantity One software (Bio-Rad).

In response to the WSSV infection, while many of the proteins showed change in the level of expression, there were few proteins newly expressed. The total protein profile of muscle showed variation in 141, 73 and 24 kDa. All of them were upregulated at 6hrs and remained expressed upto 48hrs. Among the nuclear proteins of the muscle, the 31 and 24 kDa proteins were upregulated from 12hrs onwards and remained so upto 48hrs. The 24 kDa protein was highly upregulated at 48hrs. The 29 kDa nuclear protein was upregulated from 24hrs onwards and it was highly expressed both at 24 and 36hrs. Upregulation of some of the mitochondrial proteins of the muscle were also observed. They were 70, 45, 39 and 24 kDa. The 70 kDa protein was upregulated from 6hrs onwards and 45 kDa from 12hrs, and both remained expressed upto 48hrs. The 39 kDa was upregulated from 36hrs and 24 kDa from 12hrs and both were intensely expressed at 48hrs. A new mitochondrial protein of 34 kDa was observed to be expressed in the muscle after 12 hours post infection and expressed highly at 48hrs.

The gill which is one of the main target organs of WSSV showed expressional variations in maximum number of proteins. The total protein profile of gills revealed upregulation of 77 and 64 kDa proteins from 12hrs onwards and with highest level of expression at 48hrs. Many low molecular weight fractions were found to be upregulated from 6hrs onwards. They were 55, 48, 47, 45, 40, 35 and 37 kDa proteins of which 45 kDa protein was highly expressed. The new proteins observed in gills after infection were 144 and 24 kDa where first one is expressed only at 48hrs and latter from 6hrs onwards upto 48hrs.

Many of the nuclear proteins of the gills also showed upregulation. The 146 kDa protein started upregulation from 6hrs and got highly expressed at 12hrs, whereas the 37 kDa protein was upregulated from 6hrs and later expression become feeble. Two proteins of 66 and 77 kDa size showed high upregulation from 6hrs onwards and expressed upto 48hrs. The 45 and 42 kDa proteins were up regulated from 12hrs. The 64 kDa protein which was expressed only in the infected animal appeared at 6hrs and was being upregulated upto 48hrs.

The mitochondrial proteins of gills which were upregulated after the infection were 98, 82, 72, 64, 56 and 33 kDa. Of these 98, 82, 72 and 56 kDa proteins started up regulation from 6hrs onwards upto 48hrs, while the 64 kDa and 33 kDa were upregulated from 24 and 12hrs respectively.

The shrimp hepatopancreas has been used as a monitor organ for the overall assessment of the health and well-being of the organism. The protein expressional variation in hepatopancreas was also monitored. The total protein profile of hepatopancreas showed high level of up regulation of 86, 61 and 49.5 kDa proteins at 48hrs. The 31 kDa was upregulated from 6hrs and was highly expressed at 48hrs. The new proteins observed in total protein of hepatopancreas after infection was 77 kDa which was expressed at 48hrs. The nuclear protein of hepatopancreas which showed differential expression were 45, 36, 26 and 23 kDa. The 45 kDa protein was highly expressed at 12 hrs. The 36 kDa protein was upregulated up to 12hrs, then from 36hrs showed downregulation. The 26 and 23 kDa proteins were up regulated from 6hrs onwards and expressed up to 48hrs. The new nuclear proteins observed in the hepatopancreas of infected animals were 148, 114, 82, and 47 kDa. The 148 and 114 kDa proteins were observed at 24hrs. The 82 kDa was observed from 6hrs and expressed upto 24hrs while 47 kDa was upregulated at 12 and 24hrs. Among the mitochondrial proteins of hepatopancreas the 106 kDa protein expression was upregulated up to 12hrs and then down regulated. Another 54 kDa was upregulated at 6hrs and expression became feeble in later hours. The 39 and 36 kDa proteins were up regulated at 6hrs and slowly downregulated from 24hrs onwards. The 28 kDa protein was expressed from 6hrs onwards and remained upregulated up to 12hrs post infection. The 64 kDa was a new protein observed only in infected animal and was highly expressed at 48hrs. This study will help to get a better understanding of the shrimp proteins that are modulated in the virus infection. In addition, the altered proteins reported in this study provide a rich source of information for the identification of novel proteins in shrimp antiviral response.

In **conclusion,** the present study revealed that WSSV infection in shrimp causes modulation of the expression of some of the genes participating in the innate immunity. The immune related genes like Lysozyme, C-lectin and Prophenoloxidase, Hemocyanin and Penaeidin are intensely expressed during the initial hours of viral invasion. Quantification of viral progression revealed that even though the viral copy number was maximum in hemocytes at 12hrs the viral progression in the gills increased steadily, overtaking hemocytes and other tissues as infection progressed. The second highest viral copy number was in pleopod followed by muscle. Death of the animals after infection was monitored and a correlation between survivability rate and gene

expression was observed. The animals which survived beyond 3½ days had higher expression of Lysozyme, C-lectin and Prophenoloxidase than those animals which died before 2½ days. The gene expression level can be used as a biomarker for selecting broodstocks for better resistance to WSSV infection, thus promising a role in the fight against WSSV along with other management strategies. The SDS-PAGE analysis of gills, hepatopancreas and muscle elucidated some newly expressed proteins and some highly upregulated proteins during WSSV progression. It is hoped that the data generated and the knowledge elucidated regarding shrimps response to WSSV shall be useful to further refine the strategies in shrimp farming. The knowledge regarding the immune gene expression shall be of good help in the selection of good broodstocks for seed production.

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